Invited Faculty – Extended Abstracts

3.1

Cartilage tissue regeneration

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All tissues in the body contain an extracellular matrix (ECM) that provides tissue shape and form and the ECM is an implicit part of being multicellular and having specialised tissues and organs to carry out different functions. As something labelled "structural" the ECM tends to be taken for granted when tissue functions are being thought about. However, this belies the fact that the ECM structure is essential to support most tissue functions. Support is provided by strong fibrillar components (collagens, elastin, microfibrillar proteins) with proteoglycan and glycoprotein components to ensure tissue hydration and compressive resilience. They together form the architectural scaffold of tissues, but the ECM is far from being a passive permanent structure for life, as each ECM is dynamic, with components being assembled and disassembled all the time. The capacity of the ECM for self-renewal is a major factor in tissue repair, following injury or disease. The capacity for repair can be insufficient to regenerate healthy tissue, or it can be misdirected to replace functional tissue with scar-tissue. The repair of articular cartilage presents some very specific challenges. This tissue has a limited capacity to repair sites of physical damage. This is largely because cartilage lacks blood vessels and blood vessels normally provide the route by which new cells arrive at a wound and begin a process of damage limitation and provide the cells and signals to initiate repair. Cartilage lacks blood vessels because it has a massively developed ECM that forms more than 95% of the tissue. It is the integrity of this ECM that is essential for the resilient properties of cartilage and structures like blood vessels would provide sites of focal weakness and thus compromise its function. So cartilage has evolved to be avascular and the cells within cartilage, the chondrocytes, thrive without being close to blood vessels and indeed they prefer a lower oxygen supply for their health and well-being. The health and well-being of the chondrocytes is important as they produce and slowly turnover the cartilage ECM in our joints all our lives. So to regenerate cartilage we need chondrocytes and we need them to efficiently assemble a large ECM that can provide the resilient tissue to replicate the function of articular cartilage. In these studies we investigated two main souces of cells for cartilage repair. The first was chondrocytes isolated from human OA knee cartilage removed at joint replacement and we assessed their potential to reassemble cartilage matrix in vitro with and without transduction with retroviral SOX9. The second source of cells investigated was human bone marrow stem cells, which are chondrogenic in culture and we investigated their matrix forming ability in a a new in vitro culture system.

Methods for chondrocyte isolation from human OA cartilage and retroviral transduction with SOX9 and desribed in reference 3 and 4 and methods for culture of human bone marrow stem cells are reported in reference 5.

Human OA Chondrocytes. The first strategy we investigated was to look at the potential to remake cartilage from the cells isolated from tissue removed at joint replacement. It has been established for 30 years that chondrocytes isolated from cartilage de-differentiate in monolayer culture and lose the expression of the ECM proteins that characterise the tissue (1). Our strategy was to investigate if artificially replacing the transcription factor, SOX9, which is essential for all chondrocytes (2), would be sufficient to retain the chondrocyte phenotype. Chondrocytes were transduced with retroviral SOX9 and the answer to the question was both yes and no. In monolayer culture the human chondrocytes transduced with SOX9 showed a modest increase in the expression of cartilage genes, but alone it did not cause a regain of matrix formation (3). However when the cells transduced with SOX9 were placed in 3D culture, as an aggregate of 500,000 cells, there was an additional increase in gene expression and if anabolic growth factors (TGFi¢ and IGF1) were added and low oxygen conditions were used, there was, with each change in condition, a further increase in ECM gene expression and the cells could now efficiently form a cartilage-like matrix in 2 weeks culture (4, 5). So the effects of transducing the cells with SOX9 was to potentiate their response to the other signals, physical and biological, that drive chondrocytes to make matrix. Furthermore, the results showed that human chondrocytes from an osteoarthritic joint could be driven to reform cartilage, which showed that they had not lost their capacity to make cartilage ECM due to their disease origin. The results also showed that these cells from elderly patients had the capacity to expand through many generations in monolayer culture and still retain the response to SOX9 transduction. They retained a chondrocyte identity throughout this passage in culture, as the same SOX9 transduction treatment applied to skin fibroblasts did not turn them into chondrocytes and drive them to make cartilage-like ECM (4). These studies carried an important message, that cells from elderly patients, even from diseased tissue, were still capable of generating new tissue given the appropriate biological signals and this gives hope for many strategies for for using existing tissue as a source of cells to repair or regenerate cartilage from existing tissue as a source of cells. Human Adult Bone Marrow Stem Cells. Sometimes the patient supply of cells from cartilage may not be adequate and there is great interest in bone marrow stem cells for the repair of cartilage, bone and other tissues. Simple protocols to guide the differentiation of bone-marrow stem cells into chondrocytes were developed almost 10 years ago (6). A key element was to provide a 3D culture in which there was a multicellular mass. This was typically achieved by forming a cell aggregate by lightly spinning down suspended cells into a pellet or by forming a micromass of cells and providing cues for differentiation (TGFb3 and dexamethasone). These conditions that favour chondrogenic differentiation mimic the mesenchymal cell condensation that happens during cartilage formation in early embryonic skeletogenesis. This condensation is a critical event in laying down the template for long bone formation and marks the beginning of the exclusion of blood vessels and nerves from the cartilage tissue. Bone marrow derived stem cells respond strongly to these conditions in culture and they rapidly grow and secrete and assemble a large cartilage-like ECM. We have now investigated this system more fully and improved it. The major change was to alter the configuration of cell culture by placing cells in a Transwell on a permeable support as a shallow multicellular layer rather than a spherical pellet and this provided very efficient nutrient supply both from above and from below. This resulted in much faster growth and assembly of the cartilage and the creation of tissue with significant mechanical properties in 2-4 weeks. The disc of cartilage produced in this scaffold-free culture at 2 weeks achieves more than 20 times the original mass of the cells at day 0. This rapid growth is accompanied by a very efficient incorporation of, for example, major components like aggrecan, into the assembled matrix. This is surprising as the surface area to volume ratio is much less for a sphere than a disc, so the loss from the matrix based on simple diffusion out of the tissue might be expected to be greater from a thin disc. This clearly shows that the shallow disc favours a more efficient assembly of the ECM and it is this which provides an effective barrier to passive diffusion of matrix proteins out of the tissue. The newly formed tissue does not approach the composition, or properties of cartilage as the collagen content is only 2% of the wet weight, whereas in cartilage, collagen is more than 20% of the wet weight. However it forms a tissue that is surprisingly cohesive in such a short time in culture and clearly shows the benefit of the combination of physical and biological signals to favour ECM production. This principle would seem to be important for any tissue engineering approach to tissue growth in vitro. Indeed it would suggest that the current transwell cartilage system might be improved further by cyclical loading whilst it is growing, as compressive loading is known to enhance matrix production. These issues are important in many areas of tissue formation, as the basic cohesion of cells into a tissue structure depends on the collagen organisation and cross-links to provide a scaffold with tensile properties to respond to tissue deformation. It appears unlikely that tissue formed in vitro can approach the full properties of mature tissue, which has been produced over many years, but it needs to reach minimum standards sufficient for function. The immaturity of engineered tissue like cartilage may however also be an advantage, as it is required to integrate with more mature existing tissue and an underdeveloped ECM full of cells pumping out ECM and still in the process of formation, may be capable of better tissue integration. Much of the strategies in tissue repair may thus focus on providing something adequate for immediate purpose, but capable of maturing into a stable tissue with long lasting properties. The in vivo biological and biomechanical environment may then provide the final driver for tissue adaptation. So providing an engineered tissue that is well suited to respond to such signals is an important attribute to build in to cell based therapies.

The application of current cell-based strategies for cartilage repair is limited by the relatively high cost of the individual culture of cells for each patient. It would be more ideal if such cell-based treatments were available from banks of cells for all-patient use. This would require the use of allogeneic cells such as stem cells for cartilage repair. The experience of tissue and organ transplants is that tissue matching is essential to avoid immune rejection, but the evidence for cell transplants is less clear and several recent papers on stem cells have suggested that they have very low immunogenicity (7, 8). It has even been suggested that stem cells may confer some degree of tolerance - that the patient once injected with stem cells is made tolerant to subsequent exposure to the stem cell progeny that might

otherwise be immunogenic. These issues have by no means been resolved, as it is difficult to devise non-human experiments that provide clear guidance on this issue. However, for many reasons cartilage would be a good candidate for trial, as the chondrocytes even in an immature ECM, are in an environment devoid of blood vessels and away from the circulation that contains the cells necessary to initiate an immune response. So it might be expected to be a site of low immunogenic risk, requiring minimal immuno-suppression. It is therefore an intriguing challenge to establish if stem cells from unrelated individuals can be used for the repair of tissues like cartilage, as this would open the way to their use on a much broader scale because of the economies possible with a banked cell supply; but that is a hope for tomorrow and not a promise for today.

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4.1

Genetically engineered elastin-like polypeptides for cartilage tissue regeneration

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Injectable, in situ forming scaffolds have long been of interest for promoting regeneration of focal cartilage defects. With liquid-like properties that allow mixing with cells or other factors, these materials can be injected to enable complete filling of irregularly-shaped defects and the delivery of cell or bioactive drugs that could enhance integration of newly generated tissue with the existing native tissue. Additional advantages are the opportunity for these scaffolds to contribute to preservation of mechanical integrity and maintenance of cell phenotypes. Many hydrogels capable of in situ formation or crosslinking have been evaluated for potential to promote cartilage matrix regeneration, including alginate[23], poly(ethylene oxide)[8], poly(N-isopropylacrylamide)[27], fibrin[26], hyaluronic acid[21], collagen gels[18], [33], chitosan[7],[19], poly(propylene fumarate)[28], and poly(vinyl alcohol)[24]. These polymers share the characteristics that they form three-dimensional hydrogels in situ of high water content that support rounded cell morphologies and rapid diffusion of soluble nutrients. However, other properties of these hydrogels differ including biocompatibility, degradation, surface interaction, and cell recognition characteristics. In situ forming materials also have the potential to facilitate tissue regeneration in 2nd and 3rd generation procedures for autologous chondrocyte delivery, where various biological or polymer membranes or scaffolds have been used to preserve retention of the delivered cells[6], [14].

Our laboratory has developed a class of polypeptide gels for the purpose of promoting cartilage matrix regeneration, biocompatible cell delivery, and tissue integration. ELPs consist of oligomeric repeats of the pentapeptide sequence Val-Pro-Gly-Xaa-Gly, where Xaa is any amino acid except proline[30]. This is a naturally occurring sequence in the protein elastin contained in muscle, ligaments, cartilage and numerous other soft tissues. ELPs have been shown not to illicit an antibody response upon implantation in multiple animal and human applications[31], and have been considered to be non-immunogenic. ELPs are environmentally responsive, as they exhibit a reversible inverse phase transition at a temperature (Tt) above which they undergo hydrophobic collapse and form intermolecular associations that result in an “aggregated” mass[11]. By designing the transition to occur at physiological temperatures, the environmental responsiveness of ELPs enables in situ formation and physical property changes that contribute to load-bearing and cell entrapment3-5. ELPs may be synthesized genetically, and so designed at the genetic level to control molecular weight, reactive crosslinking sites, and chemistry of the amino acid residues[16],[17]. In this paper, we review our experience with uncrosslinked and crosslinkable ELPs for an ability to entrap chondrocytes and adult stem cells, contribute to mechanical load-bearing properties, and promote matrix integration in a goat model of osteochondral defect-filling. The results illustrate the favorable characteristics and facile synthesis of an in situ forming, biocompatible hydrogel that can assist cartilage matrix regeneration in multiple ways.

Elastin-Like Polypeptide Gene Design and Synthesis. Genes have been designed to encode a wide range of ELP proteins with different guest residue sequences and combinations thereof[17]. For a given ELP, genes encoding the targeted pentapeptide sequence are first constructed from synthetic oligonucleotides, and oligomerized by recursive directional ligation17. In our laboratory, peptides have been expressed in E. coli. ELPs may be easily purified by inverse transition cycling. This procedure consists of a series of cyclic temperature changes that drive the transitioning of the ELP sequence, but not other proteins resulting from the expression. After only two sequences of temperature shifts above and below the Tt (generally between 28-34°C) the purity of the resulting ELP product has been shown to be excellent, with endotoxin levels far below FDA allowable limits for medical devices[15].

Chondrocyte and Stem Cell Encapsulation for In Vitro Culture. ELPs were designed with Val, Gly and Ala in a 5:2:3 ratio, respectively, at the guest residue position of the pentapeptide (36 kDa MW). These ELPs give rise to a target Tt of 35°C at a solution concentration of 50 mg/ml and so enable their use for in situ forming gels at physiological temperatures5. Physical characteristics of the ELPs were obtained by evaluating the rheological properties above and below the Tt using viscometry experiments. Below Tt, the apparent viscosity $|\eta^*|$ and dynamic shear modulus $|G^*|$ were sensitive to ELP concentration and exhibited a 2-fold increase in magnitude with a 4-fold increase in concentration. However, by increasing Tt to 37°C,